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Inhibitory Effect of Diphtheria Toxin on Amino Acid Incorporation in *Escherichia coli* Cell-Free System

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The mechanism of action of diphtheria toxin in an *Escherichia coli* cell-free protein-synthesizing system was examined. When the washed ribosomes were incubated with toxin before addition of messenger ribonucleic acid (RNA), peptide syntheses of ^{14}C-phenylalanine directed by polyuridylic acid or phage R17 RNA were strongly inhibited by a small amount of toxin. Whereas, if the soluble fraction (105,000 × *g* supernatant fraction) was preincubated with toxin, no effect of toxin occurred either on the induced protein synthesis or on the activity of guanosine triphosphatase even in the presence of nicotinamide adenine dinucleotide. The binding of ^{3}H-formylmethionyl-transfer RNA to *E. coli* ribosomes directed by either R17 RNA or trinucleotide AUG was also decreased by toxin. These findings suggest that diphtheria toxin may prevent the binding of messenger RNA by successfully competing with the AUG for ribosomal binding sites. Sucrose-density gradient studies support this concept by showing the decrease in binding of ^{3}H-labeled R17 RNA to *E. coli* ribosomes exposed to toxin.

Several workers have reported that diphtheria toxin inhibits protein synthesis in mammalian cell-free systems in the presence of nicotinamide adenine dinucleotide (NAD; 2, 6, 16). Recent work of Honjo et al. (9) has revealed that, in the presence of toxin, the adenosine diphosphoribose portion of NAD was transferred to aminoacyl transferase II (TFase II) obtained from rat liver, an event linked to the loss of transferase activity.

According to Collier and Pappenheimer (2) and Goor et al. (7), diphtheria toxin was unable to inhibit the incorporation of amino acid in *Escherichia coli* extracts. Johnson et al. (10) have also reported that a cell-free system of *Streptococcus faecalis* was resistant to high concentrations of diphtheria toxin. However, Goto et al. (8) and Kato (13) found that protein synthesis induced by added messenger ribonucleic acid (mRNA) in a cell-free system from *Corynebacterium diphtheriae* was readily inhibited by diphtheria toxin in the same concentrations as those found to be effective in mammalian cell-free systems, whereas protein synthesis attributable to mRNA already attached to ribosomes (endogenous protein synthesis) was not significantly affected regardless of the presence of NAD. They suggested from these findings that once mRNA is bound to bacterial ribosomes it is no longer susceptible to the mechanism of action of diphtheria toxin.

In this paper we followed up the experiments of Goto et al. (8) by using a refined *E. coli* cell-free protein-synthesizing system; to understand more fully the mode of action of diphtheria toxin, we focused mainly on the initiation of protein synthesis.

MATERIALS AND METHODS

Chemicals. ^{14}C-phenylalanine (504 mCi/mmole) was obtained from Daiichi Pure Chemicals Co. Ltd., Tokyo; L-methionine-*methyl*-^{3}H (11.1 Ci/mmole) from Radiochemical Centre, Amersham, England; folinic acid from General Biochemicals, Chagrin Falls, Ohio. Poly U was purchased from Miles Laboratories, Inc., Elkhart, Ind. Trinucleotide AUG was kindly given us by T. Ohta of the University of Tokyo.

Diphtheria toxin. Crystalline diphtheria toxin was prepared by the method of Kato et al. (11). The purified toxin contained 50 MLD per Lf and 1 Lf was equal to 2 μg of toxin.

Preparation of cell-free extracts. *E. coli* (Q13 strain) cells were harvested during exponential growth. The cell pellet was ground with quartz sand in a prechilled (−20 C) mortar. The enzymes were extracted with medium I [0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.5), 0.01 M magnesium acetate, 0.15 M NH_4Cl, 0.006 M 2-mercaptoethanol] containing 2 μg of deoxyribonuclease per ml. The *S*30 fraction was obtained by centrifuging the crude extract at 30,000 × *g* for 30 min. The *S*105

fraction was obtained by centrifuging the *S*30 fraction at 105,000 × *g* for 4 hr at 0 C. The *S*105 fraction was dialyzed against medium I at 4 C overnight. The ribosomal pellet was suspended in medium I containing 1.0 M NH_4Cl (medium II) and placed in the ice for several hours. The ribosome suspension was diluted with an equal volume of medium II and then centrifuged at 105,000 × *g* for 4 hr. The resulting supernatant fraction was used for the preparation of the initiation factors. The ribosomal pellet was washed three times with medium II by the centrifugation at 105,000 × *g* for 4 hr. The final ribosomal pellet was suspended in medium I (washed ribosomes). The initiation factors were prepared from the supernatant fluid of washed ribosomes. The supernatant fraction was precipitated by adding $(NH_4)_2SO_4$ to 70% saturation. The resulting precipitates were dialyzed against 0.01 M Tris-hydrochloride buffer (*p*H 7.5) containing 1 mM dithiothreitol at 4 C overnight. The final dialyzed solution was designated crude initiation factors.

Preparation of ^{3}H-F-methionine-transfer RNA (tRNA) and ^{14}C-phenylalanine-tRNA. The formation of ^{3}H-formylmethionyl-tRNA was carried out in a single step. The reaction mixture contained, per 4 ml, 13.5 mg of *E. coli* tRNA, 5 mg of *S*105, 200 μg of creatine kinase, and the following constituents: 25 mM Tris-hydrochloride (*p*H 7.5), 15 mM magnesium acetate, 5 mM 2-mercaptoethanol, 4 mM adenosine triphosphate (ATP), 0.5 mM cytosine triphosphate (CTP), 10 mM creatine phosphate, 0.6 mM folinic acid, 19 cold amino acids (less methionine) at a concentration of 1 mM, 0.09 mM ^{3}H-methionine. After 15 min of incubation at 37 C, the charged tRNA was extracted by the phenol method of von Ehrenstein and Lipmann (18). The final ethanol precipitate was dissolved in distilled water. The solution was passed through Sephadex G-25. The ^{3}H-formylmethionyl-tRNA fraction was collected by ethanol precipitation, and dissolved in distilled water; the final solution was adjusted to *p*H 5.0 with acetate buffer. The preparation of ^{14}C-phenylalanine-tRNA was carried out as described above with exception of the amino acids (less phenylalanine) and 0.09 mM ^{14}C-phenylalanine.

Incorporation of ^{14}C-phenylalanine. The reaction mixture contained in a total volume of 0.1 ml: 3 optical density units at 260 nm (OD_{260}) of washed ribosomes, 0.12 mg of the *S*105 fraction, either 10 μg of poly U or 31 μg of R17 RNA, 3.4 OD_{260} units of ^{14}C-phenylalanine-tRNA, 2 mM guanosine triphosphate (GTP) and its generating system, 10 mM Tris-hydrochloride (*p*H 7.5), 10 mM magnesium acetate, 150 mM NH_4Cl, and 6 mM 2-mercaptoethanol. The incubation was carried out for 20 min at 37 C and terminated by the addition of 3 ml of 5% trichloroacetic acid and heated for 15 min at 90 C. The precipitates were collected on Whatmann glass-fiber filters (GF/C), and were washed further with 15 ml of 5% trichloroacetic acid. Radioactivity was counted in 5 ml of toluene-based scintillator in Beckman scintillation counter.

Binding of ^{3}H-formylmethionyl-tRNA. Reaction mixture contained in a total volume of 0.1 ml: 10 mM Tris-hydrochloride (*p*H 7.5), 10 mM magnesium acetate, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol, 1 mM GTP, 3 OD_{260} units of washed ribosomes, 0.88 OD_{260} units of ^{3}H-formylmethionyl-tRNA, 7.5 μg of initiation factors and either 31 μg of R17 RNA or 1 OD_{260} unit of AUG. The incubation was carried out for 15 min at 37 C, and the reaction mixture was immediately diluted with 3 ml of cold medium I. The samples were filtered through membrane filters (HAWP; pore size, 0.45 μm; Millipore Corp.) and were further washed with cold medium I. Radioactivity was counted in 5 ml of toluene-based scintillator in Beckman scintillation counter.

Assay of guanosine triphosphatase (GTPase). The activity of ribosome-dependent GTPase of G factor was determined by the following reaction mixtures which were assembled in a total volume of 0.2 ml. Each mixture contained 50 mM Tris-hydrochloride (*p*H 8.0), 10 mM $MgCl_2$, 150 mM NH_4Cl, 10 mM 2-mercaptoethanol, 0.04 mmole of NAD, 0.02 μmole of ^{32}P-GTP (gamma-labeled), 3 OD_{260} units of washed ribosomes, and either 0.012 mg of *S*105 enzymes or 5×10^{-3} units of crystalline G factor to be assayed. The incubation was carried out for 20 min at 37 C. The liberation of inorganic phosphate from labeled GTP was determined by the method of Conway and Lipmann (4).

RESULTS

Effect of diphtheria toxin on poly U-directed incorporation of ^{14}C-phenylalanine. Toxin at a concentration of even 0.4 μg/ml strongly inhibited the incorporation of poly U-directed ^{14}C-phenylalanine, when toxin and washed *E. coli* ribosomes were incubated for 2 min at 37 C before the addition of poly U (Table 1, Fig. 1). Whereas, in preliminary experiments, we found that toxin did not affect the induced protein synthesis, if toxin was added to the reaction mixture after the addition of poly U. Furthermore, when *S*105 fraction was incubated with toxin before the addition of poly U and ribosomes, significant

TABLE 1. *Effect of diphtheria toxin on the incorporation of ^{14}C-phenylalanine into protein in a cell-free system from E. coli*[a]

Reaction	Toxin added	Incorporation	Inhibition
	μg/ml	counts per min per ml	%
1	None	11,316	
2	400	2,588	77
3	40	1,040	92
4	4	1,798	84
5	0.4	1,484	87

[a] In each reaction, ribosomes and toxin were incubated for 2 min at 37 C before the addition of poly U (except reaction 1). The other conditions were as described in the text.

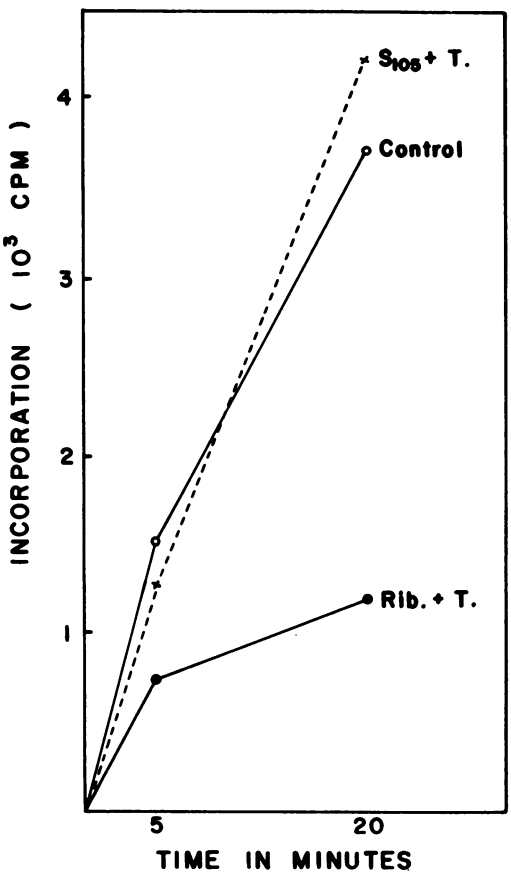

FIG. 1. *Effect of diphtheria toxin on poly U-directed incorporation of ^{14}C-phenylalanine. Each reaction mixture (0.1 ml) was incubated at 37 C, and the ^{14}C-phenylalanine incorporated into protein was assayed as described in the text. Control was incubated without toxin (○). Toxin (40 μg) and washed ribosomes (3 OD_{260}) were preincubated before the addition of poly U (●). Toxin (40 μg) and S105 fraction (0.12 mg) were preincubated (×). The incorporations of ^{14}C-phenylalanine into both ribosomes and S105 fraction were not detected in the absence of poly U.*

inhibition of protein synthesis did not occur in the reaction mixture. These findings were in complete accord with the recent experiments of Goto et al. (8) and Kato (13). Therefore, these observations suggest that the functioning of messenger RNA (mRNA) previously bound to bacterial ribosomes is not susceptible to the inhibition of protein synthesis by diphtheria toxin.

Effect of diphtheria toxin on R17 RNA-directed incorporation of ^{14}C-phenylalanine. Toxin strongly inhibited the peptide formation of ^{14}C-phenylalanine directed by either phage R17 RNA or synthetic poly U (Table 2).

Effect of diphtheria toxin on E. coli polysomes. To determine whether toxin caused any structural alterations of polysomes, sucrose-density gradient centrifugation was used; results are shown in Fig. 2. It is clear from the OD_{260} readings in the two gradient profiles that toxin did not accelerate the breakdown of polysomes. This result agreed with the report of Collier (3). He has clearly demonstrated that toxin did not break down polysomes from rabbit reticulocyte to single ribosomes.

Effect of diphtheria toxin on GTPase activity. Since toxin inhibits the activity of TFase II in the presence of NAD in mammalian cell-free system, we would expect to find the effect of toxin on ribosome-dependent GTPase activity of G factor in *E. coli* which corresponds to TFase II. The result is given in Table 3, which shows that the activity of GTPase in the presence of NAD is not inactivated by toxin. However, the resistance to toxin of the activity of bacterial transferase is not yet clearly explained.

Effect of diphtheria toxin on the binding of ^{3}H-formylmethionyl-tRNA to ribosomes. Recently insight has been obtained into the mechanism of chain initiation in *E. coli*. Adams and Capecchi (1) and Webster et al. (19) found that formylmethionyl-tRNA was incorporated into the peptide chain initiation when a ribosome was attached to a single mRNA such as AUG codon. We examined the effect of toxin on the binding of ^{3}H-formylmethionyl-tRNA to ribosomes directed by both R17 RNA and trinucleotide AUG. Table 4 shows that 4 μg of toxin strongly inhibited the bindings of ^{3}H-formylmethionyl-tRNA to ribosomes directed by both R17 RNA

TABLE 2. *Effect of diphtheria toxin on R17 RNA and poly U-directed incorporation of ^{14}C-phenylalanine*

Reaction[a]	mRNA	Toxin added	Incorporation	Inhibition
		μg	*counts/min*	%
1	R17 RNA[b]	None	177	
2		2	38	80
3		4	25	86
4	Poly U	None	3,498	
5		4	620	82

[a] To reactions 1, 2, and 3 were added 7.5 μg of initiation factors besides reaction mixtures described in the text. Reactions 2, 3, and 5: washed ribosomes and toxin were incubated for 2 min at 37 C before the addition of messenger RNA (mRNA); reactions 1 and 4: only ribosomes were preincubated.

[b] R17 RNA was kindly given us by H. Matsuzawa of The University of Tokyo.

and trinucleotide AUG, when the ribosomes were incubated with toxin before onset of the reaction.

The inhibition of binding of ^{3}H-labeled R17 RNA to ribosomes by toxin was clearly demonstrated in Fig. 3 by using the centrifugation through a linear sucrose gradient. In the absence of initiation factors, ^{3}H-R17 RNA was not associated with the 70*S* ribosomes (Fig. 3a). In the presence of initiation factors, however, a large portion of ^{3}H-R17 RNA was bound in the 70*S* ribosome region (Fig. 3b). When the ribosomes were preincubated with toxin, the binding of ^{3}H-R17 RNA in 70*S* region was decreased by about 65% as compared with control (Fig. 3c). These findings lead to the conclusion that toxin does not affect the activity of initiation factors but toxin interfers with the binding of mRNA to ribosomes.

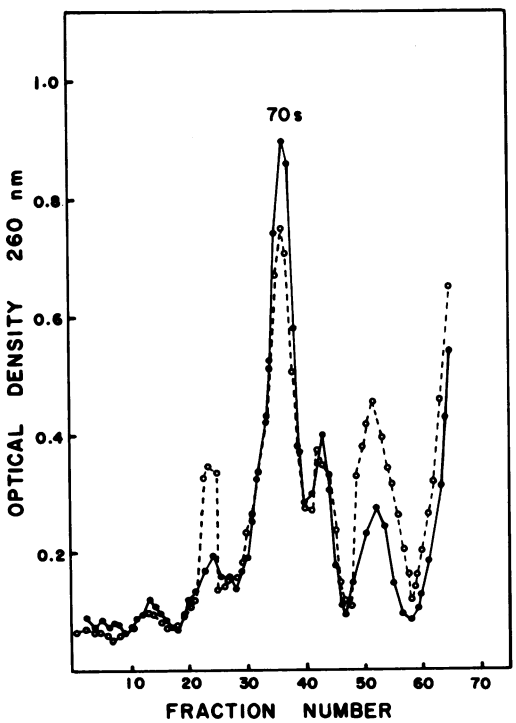

FIG. 2. *Effect of diphtheria toxin on patterns of E. coli polysomes. Polysomes were prepared from E. coli spheroplasts by the method of Godson (5). One milliliter of polysomes (2 mg) and toxin (50 μg) were preincubated for 10 min at 37 C and were layered on top of 30 ml of 15 to 30% linear sucrose gradient supplemented with medium I (●). Control: without toxin (○). The gradients were centrifuged for 7.5 hr at 24,000 rev/min by Spinco SW 25 rotor at 4 C.*

TABLE 3. *Effect of diphtheria toxin on GTPase activity*

Reaction	Enzyme source	Toxin added	NAD	^{32}P-GTP hydrolysis
		μg	*nmole*	*nmole*
1	*S*105	None	None	0.86
2		4	None	0.86
3		4	0.04	0.90
4	G factor[a]	None	0.04	0.82
5		4	0.04	0.84

[a] Crystalline G factor was kindly given us by Y. Kaziro of The University of Tokyo.

TABLE 4. *Effect of diphtheria toxin on the binding of ^{3}H-formylmethionyl-transfer RNA to ribosomes*

Reaction[a]	Messenger RNA	Toxin added	Bound[b]	Inhibition
		μg	*counts/min*	%
1	R17 RNA	None	4,840	
2		2	3,367	30
3		4	948	80
4	AUG	None	792	
5		2	262	67
6		4	103	87

[a] Reactions 2, 3, 5, and 6: washed ribosomes and toxin were preincubated for 2 min at 37 C; reactions 1 and 4: only ribosomes were preincubated. The conditions were described in the text except that in reactions 4, 5, and 6, the reaction mixture was incubated for 25 min at 25 C.

[b] The value was corrected by subtraction of that in the absence of messenger RNA.

DISCUSSION

This paper has dealt with studies undertaken to determine the effect of diphtheria toxin in *E. coli* extracts when newly induced protein synthesis is stimulated by mRNA.

In mammalian cell-free system, it has been known that the activity of TFase II is inactivated by toxin in the presence of NAD (2, 6). However, our experiments (Table 3) showed that GTPase activity of *E. coli* G factor which corresponds to TFase II was not inactivated by toxin even in the presence of NAD. Although the requirements for protein synthesis in both mammalian and bacterial cell-free systems are remarkably similar, the difference in sensitivity to chloramphenicol in both cell-free systems has been well known. Rendi and Ochoa (17) suggested the possibility that the difference in sensitivity might be related to functional or structural differences in both ribosomes. Johnson et

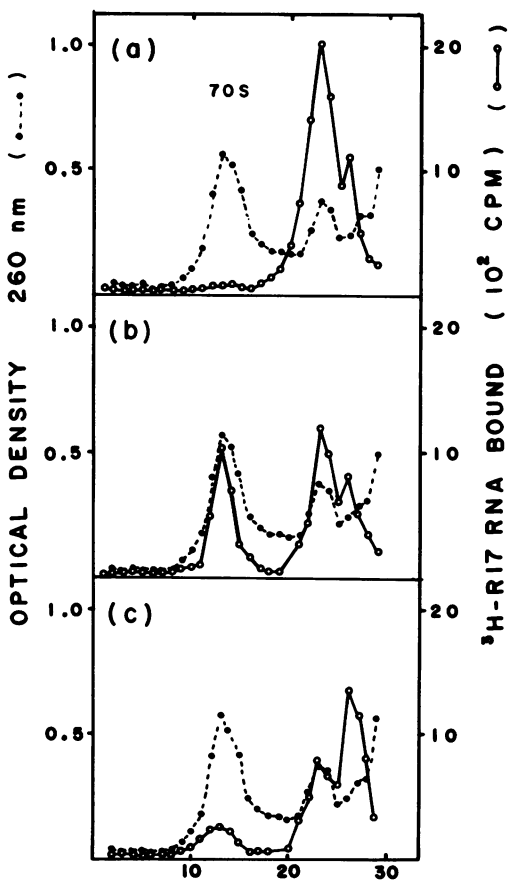

FIG. 3. *Effect of diphtheria toxin on the binding of ^{3}H-R17 RNA to ribosomes. The reaction mixture for initiation complex formation contained in a final volume of 0.12 ml: 10 mM Tris-hydrochloride (pH 7.5), 10 mM magnesium acetate, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol, 1 mM GTP, 4.5 OD_{260} units of washed ribosomes, 7.5 μg of initiation factors, 0.07 mg of ^{3}H-R17 RNA* (8.8 × 10^3 counts/min), 1 OD_{260} unit of tRNA (formylated). The reaction mixture in part a was the same as part b except that initiation factors were omitted. In the reaction mixture of part c, ribosomes were incubated with toxin (4 μg) for 3 min at 37 C before the addition of R17 RNA. Each reaction mixture was incubated for 15 min at 37 C and was immediately layered on top of 4.6 ml of 15 to 30% linear sucrose gradient supplemented with medium I. The gradients were centrifuged for 4 hr at 38,000 rev/min by Spinco SW 39 rotor at 4 C. The gradient was collected in about 30 fractions. To each fraction, the absorbancy was measured at 260 nm; 5% trichloroacetic acid was then added, and the resulting suspensions were filtered through Whatman glass fiber filters (GF/C) and washed by 5% acid. The filter was dried and counted in 5 ml of toluene-based scintillator in a Beckman scintillation counter. *^{3}H-labeled R17 RNA was kindly given us by H. Matsuzawa of the University of Tokyo.*

al. (10) and Maulkin and Lipmann (15) suggested that the resistance of bacterial cell-free protein synthesizing systems to diphtheria toxin may reflect basic differences between TFase II from mammalian and bacterial sources. Since we, in fact, demonstrated that toxin did not affect the activity of *E. coli* G factor, it seems to mean that the mechanism of action of toxin may not be precisely the same in mammalian and in bacterial systems.

In kinetic studies on the effect of diphtheria toxin on the induced protein synthesis by poly U, toxin did not affect ^{14}C-polyphenylalanine synthesis unless the ribosomes were preincubated with toxin. These observations were consistent with the results of Collier and Pappenheimer (2), Johnson et al. (10), and Goto et al. (8). However, the relatively low concentration of toxin (0.4 μg/ml) strongly inhibited the induced protein syntheses directed by both poly U and R17 RNA, when the ribosomes were preincubated with toxin for 2 min. We considered from the experiments described above that, in bacterial systems, protein synthesis attributable to mRNA already attached to ribosomes is not significantly affected by diphtheria toxin and that the effect of toxin is localized to an aspect of ribosomal function. Since toxin does not break down *E. coli* polysomes which are associated with mRNA (Fig. 2), toxin may act on the initiation step of protein synthesis.

Much is known of the process of the initiation of protein synthesis in bacteria (14). The 30*S* ribosomal subunit, in conjunction with initiation factors, interacts with mRNA. This complex in turn binds initiator formylmethionyl-tRNA and associates with the 50*S* ribosomal subunit to form the completed initiation complex. In the absence of initiation factors, neither mRNA nor initiator formylmethionyl-tRNA is bound to ribosomes. The binding of initiator ^{3}H-formylmethionyl-tRNA to ribosomes directed by either R17 RNA or trinucleotide AUG was also inhibited by toxin, when ribosomes and toxin were preincubated. Sucrose-density gradient studies supported these observations by showing the decrease in binding of ^{3}H-labeled R17 RNA to 70*S* ribosomes in the presence of toxin (Fig. 3).

Kato (13) and Kato and Watanuki (12) have reported that ^{125}I-labeled toxin bound to *E. coli* ribosomes in a molal ratio of one to one, preferably to 30*S* ribosomal subunit, and especially bound to 16*S* ribosomal RNA which is a constituent element of 30*S* ribosome. Since it has been known that mRNA attaches to 30*S*

ribosome, our findings on the mechanism of action of diphtheria toxin on protein synthesis in bacterial systems can be explained by the hypothesis that toxin successfully competes with mRNA for ribosomal binding sites, thereby blocking the initiation step of protein synthesis.

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